



Year: 2014

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DOI: <https://doi.org/10.3389/fnagi.2014.00237>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-99312>

Journal Article

Published Version



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Originally published at:

Keskitalo, Salla; Farkas, Melinda; Hanenberg, Michael; Szodorai, Anita; Kulic, Luka; Semmler, Alexander; Weller, Michael; Nitsch, Roger M; Linnebank, Michael (2014). Reciprocal modulation of A 42 aggregation by copper and homocysteine. *Frontiers in Aging Neuroscience*:6:237.

DOI: <https://doi.org/10.3389/fnagi.2014.00237>



Reciprocal modulation of A β 42 aggregation by copper and homocysteine

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Hyperhomocysteinemia is a risk factor for Alzheimer's disease (AD). Both homocysteine (Hcy) and amyloid β (A β), which accumulates in the brain of AD patients, bind copper. Aim of this study was to test the hypothesis that the association of Hcy and AD results from a molecular interaction between Hcy and A β that is mediated by copper. We established a microtiter plate format thioflavin T aggregation assay to monitor A β 42 fibrillization. Copper (5 μ M) completely prevented A β 42 (5 μ M) fibrillization. Homocysteine in the absence of copper did not impact A β 42 fibrillization, but physiological concentrations of Hcy (10–100 μ M) attenuated the inhibitory effect of copper on A β 42 fibril formation. These results were qualitatively confirmed by electron microscopy, which did not reveal morphological differences. To compare the toxicity of fibrillar and non-fibrillar A β 42 exposed to copper or Hcy, rat primary cortical neurons were treated *in vitro* with 5 μ M A β 42 for 72 h. After incubation with 5 μ M A β 42 that had been aggregating in the absence of Hcy or copper, cell viability was reduced to 40%. Incubation with 5 μ M A β 42, in which fibril formation had been prevented or reverted by the addition of 5 μ M copper, resulted in cell viability of approximately 25%. Accordingly, viability was reduced to 25% after incubation with 5 μ M monomeric, i.e., non-fibrillized, A β 42. The addition of Hcy plus copper to 5 μ M A β 42 yielded 50% viability. In conclusion, copper prevents and reverts A β fibril formation leading rather to formation of lower order oligomers or amorphous aggregates, and Hcy reduces these effects. Such mechanisms may explain the association of hyperhomocysteinemia and AD, leading to novel therapeutic strategies in the prevention and treatment of this disease.

Keywords: homocysteine, Alzheimer's disease, copper, A β , cytotoxicity, primary neurons

INTRODUCTION

Alzheimer's disease (AD) is a multifactorial neurodegenerative condition constituting the majority of dementias. Primary feature of AD is neuronal cell loss in the hippocampus and cerebral cortex, areas involved in memory and cognition (Bernardo et al., 2007; Kim et al., 2008). Histopathological characteristics are depositions of amyloid plaques, comprising extracellular accumulations of fibrillar amyloid β (A β)-peptide, and the formation of intracellular neurofibrillary tangles composed of hyperphosphorylated tau (P-tau; Hooijmans et al., 2009; Kim and Tsai, 2009). A β is produced by cleavage of the amyloid precursor protein (APP) by β -secretase (BACE-1) and γ -secretase, which is comprised of four proteins: presenilin (PS) -1 or -2, PEN, Aph-1 and Nicastrin. Cleavage of APP by β -secretase results in a N-terminal soluble fragment and a C-terminal fragment that is further cleaved by γ -secretase resulting in A β peptides. Missense mutations in either APP or PS-1 can cause accumulation of A β in hereditary AD. The mechanism leading to A β accumulation in the majority of sporadic AD patients is unclear (Mare et al., 2007). Extracellular aggregation of the A β -peptide is considered a

central and causative phenomenon of AD (Yoshiike et al., 2001; Hooijmans et al., 2009; Zatta et al., 2009; Finder et al., 2010). However, in AD patients, A β is also present in elevated amounts within the degenerating neurons, and this may contribute to cell death (Hasegawa et al., 2005).

In vivo A β has two predominant forms: A β 1-40 and A β 1-42 with two additional hydrophobic residues at the carboxyterminus. A β 1-40 is the main soluble species, whereas A β 1-42 is the predominant species found in amyloid plaques. The latter is more toxic to neurons and is considered the most amyloidogenic species, most likely responsible for the neuropathology in AD (Hasegawa et al., 2005; Mare et al., 2007; Finder et al., 2010). Amyloid β aggregation is believed to happen in phases: first, A β monomers associate into soluble oligomers that then form insoluble oligomers (initial slow nucleation or "seeding"), generating protofibrils, and fibrils (Finder and Glockshuber, 2007; Töugu et al., 2009).

Whether A β forms fibrils *in vitro* in the presence of copper and the nature of these fibrils is currently a subject of debate. Main question is the accelerating or preventing role of copper

in amyloid fibril formation, and whether this role is dependent on Cu^{2+} or $\text{A}\beta$ concentration and stoichiometry. It has been presented that sub-stoichiometric concentrations of Cu^{2+} accelerate amyloid fibril formation, and supra-stoichiometric concentrations of Cu^{2+} prevent fibrillization (Viles, 2012). There are several studies where Cu^{2+} was reported to inhibit fibril formation and rather form amorphous aggregates (Yoshiike et al., 2001; Raman et al., 2005; Töugu et al., 2009; Innocenti et al., 2010). On the contrary, the opposing arguments rely mainly on the study of Sarell et al. (2010) where the substoichiometric levels of Cu^{2+} were shown to accelerate fibril formation of $\text{A}\beta$. A recent study of Mold et al. (2013) addresses this dilemma by fluorimetry and transmission electron microscopy (TEM). In this study they show that Cu^{2+} , independent of stoichiometry, prevented the formation of ThT-positive amyloid fibrils of $\text{A}\beta 42$.

Amyloid plaques are composed of fibrillar $\text{A}\beta$, small amounts of other proteins and transition metals like copper and zinc (Töugu et al., 2009). Several studies have shown that homeostasis of the transition metals copper and zinc can greatly influence $\text{A}\beta$ misfolding and plaque formation. Furthermore, restoring metal ion homeostasis dissolved $\text{A}\beta$ plaques in mice and delayed cognitive deficits in AD patients (Zatta et al., 2009). Thus, an interaction between $\text{A}\beta$ and copper may be involved in AD pathology (Klevay, 2007a,b).

We have previously shown that homocysteine (Hcy) binds copper, and that this may be an important mechanism of the neurotoxicity of Hcy, as the presence of Hcy can lead to deficiency of copper-dependent enzymes like cytochrome-C-oxidase (White et al., 2001; Apostolova et al., 2003; Linnebank et al., 2006). Hcy is a non-proteinogenic sulfhydryl-containing amino acid formed as an intermediate in the metabolism of methionine (Hasegawa et al., 2005; Bernardo et al., 2007; Kim et al., 2008). Deficiencies of vitamin B12 or folate, common conditions in the elderly, can lead to hyperhomocysteinemia, which is a risk factor for cardio- and cerebrovascular diseases as well as neurodegenerative disorders such as AD (White et al., 2001; Irizarry et al., 2005; Linnebank et al., 2006; Bernardo et al., 2007; Kim et al., 2008). In hyperhomocysteinemic patients, blood copper levels are elevated, possibly due to binding to increased amounts of Hcy (Apostolova et al., 2003; Linnebank et al., 2006). In cell culture, Hcy sensitizes neurons to $\text{A}\beta$ toxicity by induction of intraneuronal $\text{A}\beta$ accumulation due to speculative mechanisms (Hasegawa et al., 2005). In addition, hyperhomocysteinemia increases $\text{A}\beta$ production in rats, probably through enhanced expression of γ -secretase and APP phosphorylation, placing hyperhomocysteinemia upstream of increased $\text{A}\beta$ production (Zhang et al., 2009). In this study, we aimed at modelling the interaction between copper, Hcy and $\text{A}\beta$ fibril formation.

MATERIALS AND METHODS

ORIGIN OF REAGENTS

All reagents were ultra pure quality and purchased from Sigma-Aldrich (Buchs, Switzerland) unless otherwise indicated. DL-Homocysteine was minimum 95% titration (Sigma-Aldrich). Recombinant $\text{A}\beta 42$ peptide was purchased as a 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) film, and His6Ala (H6A) mutated

recombinant $\text{A}\beta 42$ and scrambled recombinant $\text{A}\beta 42$ as trifluoroacetic acid (TFA) film from rPeptide (Bogart, Georgia, USA). Solutions were prepared in fresh MilliQ-water.

PREPARATION OF $\text{A}\beta 42$ PEPTIDE STOCKS

To ensure homogenous preparation of the $\text{A}\beta 42$ peptide, 1 mg of recombinant peptide HFIP or TFA film was distributed in 50 μg aliquots. All peptides were aliquotted with the same procedure. After addition of 200 μL HFIP to 1 mg peptide, the solution was shortly sonicated, transferred into a Protein LoBind tube (Eppendorf, Hamburg, Germany), and the solvent was evaporated with a constant stream of nitrogen. The peptide film was resuspended in 1 ml of HFIP and, after short vortexing and sonication, dispensed in 50 μg aliquots. HFIP was evaporated under a stream of nitrogen, aliquots were snap-frozen, and stored at -80°C until use as described previously (Wood et al., 1996; Stine et al., 2003).

PREPARATION OF FRESH $\text{A}\beta 42$ WORKING SOLUTION

For assays, one $\text{A}\beta 42$ peptide aliquot was dissolved in 44.4 μL 10 mM NaOH, pH 12, to yield a stock solution of approximately 250 μM . The aliquot was vortexed, sonicated, vortexed again shortly, spun down and placed on ice until use. The resuspension of the $\text{A}\beta 1-42$ film in 10 mM sodium hydroxide was adapted from Teplow (2006). The low NaOH concentration reassured the rapid pH neutralization to 7.4 upon dilution into the experimental buffer (Teplow, 2006).

The concentration of the $\text{A}\beta 42$ solution was determined via absorbance at $\lambda = 280$ nm measured with NanoDrop UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington Delaware, USA). Concentration was calculated using a molar extinction coefficient of $\epsilon = 1730 \text{ M}^{-1} \text{ cm}^{-1}$ (Finder et al., 2010).

THIOFLAVIN T AGGREGATION ASSAYS

To study amyloid fibril formation, 5 μM $\text{A}\beta 42$ peptide was mixed with 50 μM Thioflavin T (ThT) in 10 mM sodium phosphate solution, 500 mM NaCl, and 0.1 mM HCl to a final volume of 100 μL . Different concentrations of ZnCl_2 , CuCl_2 and Hcy were added to selected samples after 0 or 120 min of measurement, respectively. Samples were incubated in a flat bottom microtiter plate, and the increase in ThT fluorescence was measured via top-beam irradiation ($\lambda_{\text{Ex}} = 450$ nm, $\lambda_{\text{Em}} = 510$ nm) with a lamp energy of 5000 (arbitrary unit) and a counting time of 0.1 s by Berthold Mithras LB 940 (Berthold Technologies GmbH, Regensburg, Switzerland). Values were recorded every 2 min with constant orbital shaking at slow speed between the measurements. Temperature was controlled to 30°C .

CYTOTOXICITY

Rat primary cortical neuron cultures were prepared as described (Finder et al., 2010). Neurons were plated in Neurobasal media (GIBCO, Invitrogen, Basel, Switzerland) with B-27 supplement (GIBCO) and L-glutamine (GIBCO) on poly-L-ornithine pre-coated 96-well plates at a density of approximately 10,000 cells per well. Cultures were maintained in a humidified 7% CO_2 incubator. Primary cortical neuron cultures were treated with $\text{A}\beta$ -fibrils

on day 6 *in vitro*. For cytotoxicity assessment, aggregation reactions were performed with 10 times higher concentrations and in the absence of ThT. Thioflavin T does not affect aggregation kinetics, but influences cytotoxicity measurements (Finder et al., 2010). Otherwise reaction parameters were as described above.

After reaching aggregation plateau (after 3 h), fibril suspensions were transferred in 1:10 (v/v) ratio in relation to cell culture medium in the wells. Final concentrations on the cells were: 5 μ M A β , 5 μ M CuCl₂ and 50 μ M Hcy. Reaction mixture without A β , CuCl₂ or Hcy was used as negative control. Just before treatment with diluted fibrils, half of the culture medium on cells was aspirated and replaced with fresh Neurobasal medium. Assays were performed minimum as triplicates. After 72 h cell viability was quantified by a colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Keskitalo et al., 2007). Briefly, treatment medium was removed from cells and replaced by Neurobasal medium with MTT. After incubation for 2 h, lysis buffer (10% SDS, 10 mM HCl) was added for the incubation of cell cultures at 37°C overnight. Absorbance was measured on the next day (Berthold Mithras LB 940, Berthold Technologies GmbH), and relative survival to control (reaction mixture without A β , CuCl₂ or Hcy) was calculated.

CELL MORPHOLOGY BY IMMUNOFLUORESCENT STAINING

Approximately 100,000 rat primary cortical neurons were seeded in 24-well plates onto glass coverslips pre-coated with poly-L-ornithine in water. On *in vitro* day 6, a 24 h-incubation with the 1:10 diluted fibrils was started. As in the MTT-assay, aggregation reactions were performed with 10 times higher reaction concentration and without ThT, half of the culture medium on cells was aspirated just before adding the aggregates. Due to the aggregation assay results and the physiologically occurring Hcy levels, we chose samples incubated with 5 μ M A β , 5 μ M CuCl₂ and 50 μ M Hcy to be shown in the results. Cells were stained minimum as duplicates. 5 μ M non-fibrillar A β 42 was used as a control.

After incubation with the fibrils the cells were fixed for 15 min at room temperature with 4% paraformaldehyde in PBS. The coverslips were rinsed with PBS, and washed three times with 0.05% Triton X-100 in TBS for 10 min each. After blocking with 5% goat serum (Millipore, Zug, Switzerland), 5% horse serum (GIBCO) and 0.2% Triton X-100 in TBS for 60 min, the coverslips were incubated with primary antibodies in blocking buffer (anti-MAP2 1:1500, Synaptic Systems, Germany; 1:100 anti-human APP 6E10, Covance, Princeton, New Jersey, USA) in a humidified chamber overnight at 4°C. On the following day coverslips were washed three times with 0.05% Triton X-100 in TBS for 10 min each, blocked for 30 min at room temperature in blocking solution, and incubated with 1:300 diluted secondary antibodies (anti-rabbit Alexa488 (Invitrogen) and anti-mouse Cy3 (Jackson ImmunoResearch, West Grove, Pennsylvania, USA)) in blocking buffer for 2 h at room temperature. After washing, cell nuclei were stained with 2-(4-aminophenyl)-1H-indole-6-carboxamide (DAPI), and coverslips were mounted on glass slides with Hydromount (Chemie Brunschwig, Basel, Switzerland). Staining was examined using a Zeiss ImagerZ1 microscope (Zeiss, Oberkochen, Germany). All images were taken with a 20 \times objective.

NEGATIVE-STAIN ELECTRON MICROSCOPY OF AGGREGATES

Aliquots of the aggregation reactions without ThT were analyzed, when plateau was readily reached after 4 h agitation at 30°C. 3 μ l of each sample was adsorbed to 300 mesh carbon-coated copper grids for 1 min and stained with 2% uranyl acetate in water for 15 s three times. After staining grids were washed with water and allowed to dry before TEM. TEM was performed on a Philips CM 12 microscope at 100 MeV.

STATISTICS

Statistical analysis was run using IBM SPSS Statistics 20 (IBM, Armonk, New York, USA), and significance was calculated using one-way ANOVA with Bonferroni's *post hoc* test for multiple comparisons. Statistical significance was considered as $p < 0.05$.

All experiments were repeated three times with $n \geq 3$ samples for each experimental condition.

RESULTS

HOMOCYSTEINE AND A β 42 COMPETE FOR CuCl₂, BUT NOT FOR ZnCl₂, IN ThT AGGREGATION ASSAY

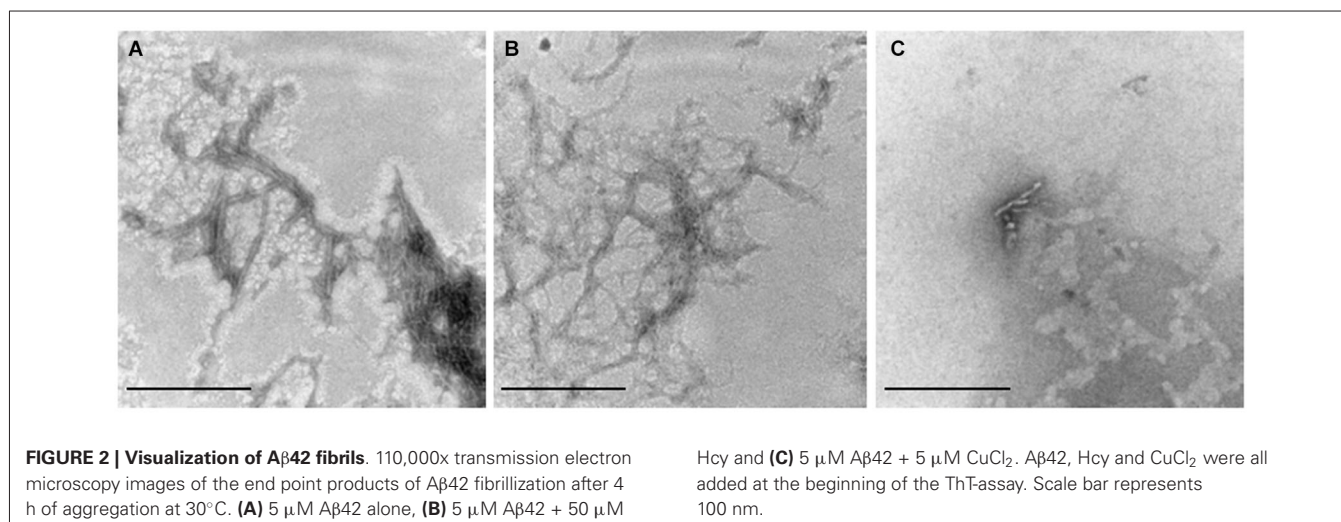
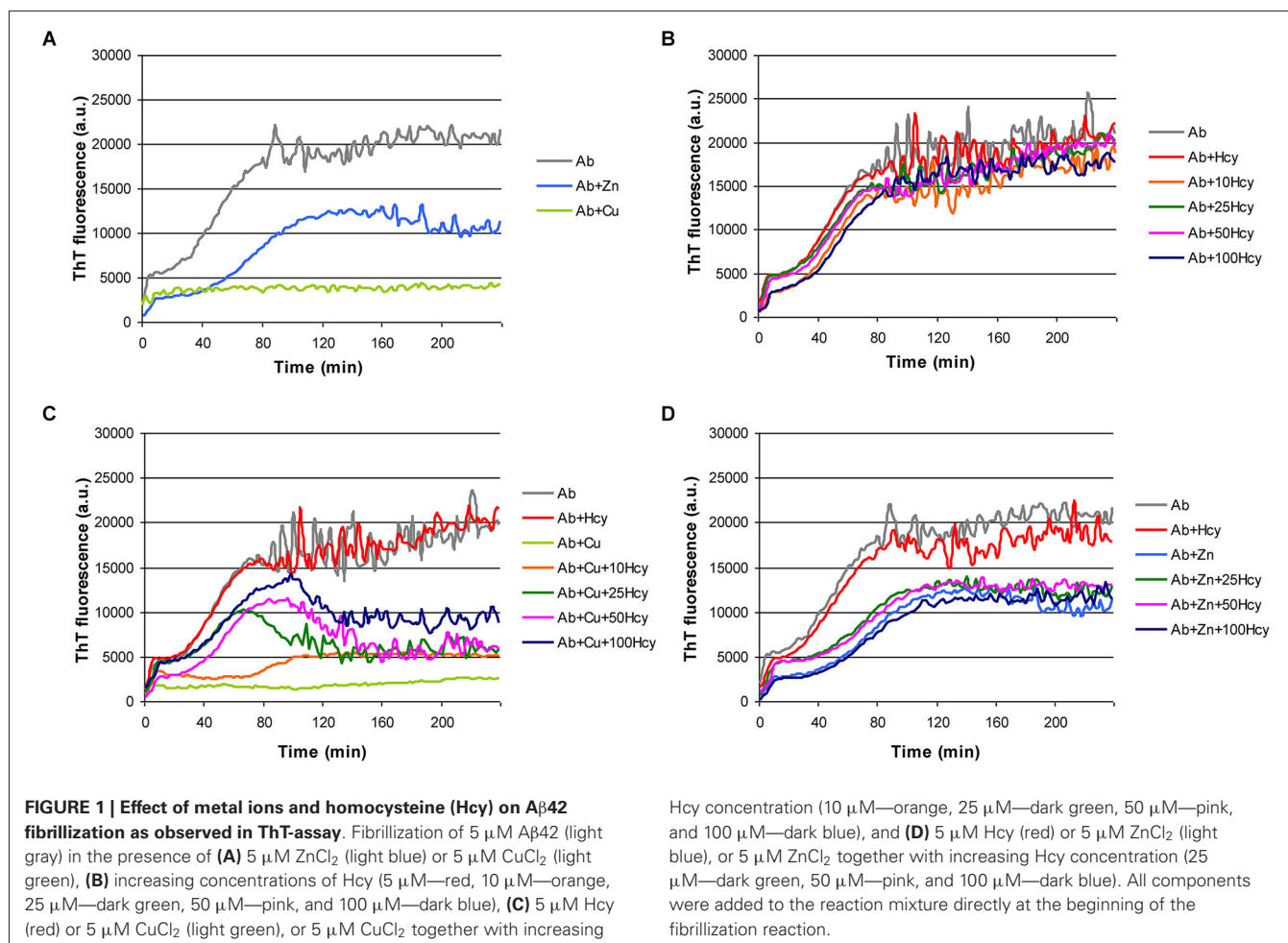
We established a microtiter plate format ThT aggregation assay for fast and reproducible monitoring of A β 42 fibrillization in the presence of Hcy and the transition metals copper and zinc. As previously reported, copper and zinc inhibited the formation of ThT reactive beta-sheet structures of A β (Yoshiike et al., 2001; House et al., 2004). In our experiments ZnCl₂ reduced A β 42 fibrillization by extending the lag phase, slightly decreasing the slope, and diminishing the final plateau (Figure 1A). CuCl₂ (5 μ M) completely prevented A β 42 (5 μ M) fibrillization (Figure 1A). Homocysteine alone at increasing concentrations had no effect on A β fibril formation (Figure 1B), but concentration-dependently reduced the inhibitory effect of CuCl₂ on A β 42 fibrillization (Figure 1C). No such interaction on A β 42 aggregation was observed between Hcy and ZnCl₂ (Figure 1D).

HOMOCYSTEINE DOES NOT ALTER A β 42 FIBRIL MORPHOLOGY

To decide whether Hcy or copper have qualitative effects on A β aggregation, we analyzed the morphology of the aggregates of 5 μ M A β 42, 5 μ M A β 42 plus 50 μ M Hcy and 5 μ M A β 42 plus 5 μ M CuCl₂ by TEM after 4 h aggregation (Figure 2). TEM images confirmed the observations from ThT aggregation assays that Hcy alone does not alter A β 42 fibrillization, as the A β 42 fibrils formed in the presence of Hcy were alike to fibrils formed without Hcy showing a high number of mature fibrils (Figures 2A,B). In the presence of CuCl₂, only few aggregates were found with decreased fibril length and complexity (Figure 2C).

TOXICITY OF A β 42 FIBRILS IS INCREASED IN THE PRESENCE OF CuCl₂ AND DECREASED IN THE PRESENCE OF HOMOCYSTEINE OR HOMOCYSTEINE PLUS CuCl₂

To be able to conclude whether cytotoxicity of mixtures of copper, Hcy and A β is caused by changes in the fibrillar status of A β 42, we examined the cytotoxic effects of CuCl₂, Hcy and the two together without A β 42 (Figure 3A). Rat primary cortical neurons were treated on day 6 *in vitro* for 72 h with increasing concentrations of CuCl₂ (0.5–5.0 μ M), Hcy (5–50 μ M) or CuCl₂



plus Hcy in incubation mixtures previously incubated for 4 h at 30°C. No significant toxicity was observed in neurons treated with the selected concentrations of CuCl₂ or Hcy alone. In line

with previous results, the co-incubation of Hcy plus CuCl₂, i.e., with homocysteine/copper-complexes, showed a concentration-dependent toxicity (White et al., 2001; Linnebank et al., 2006).

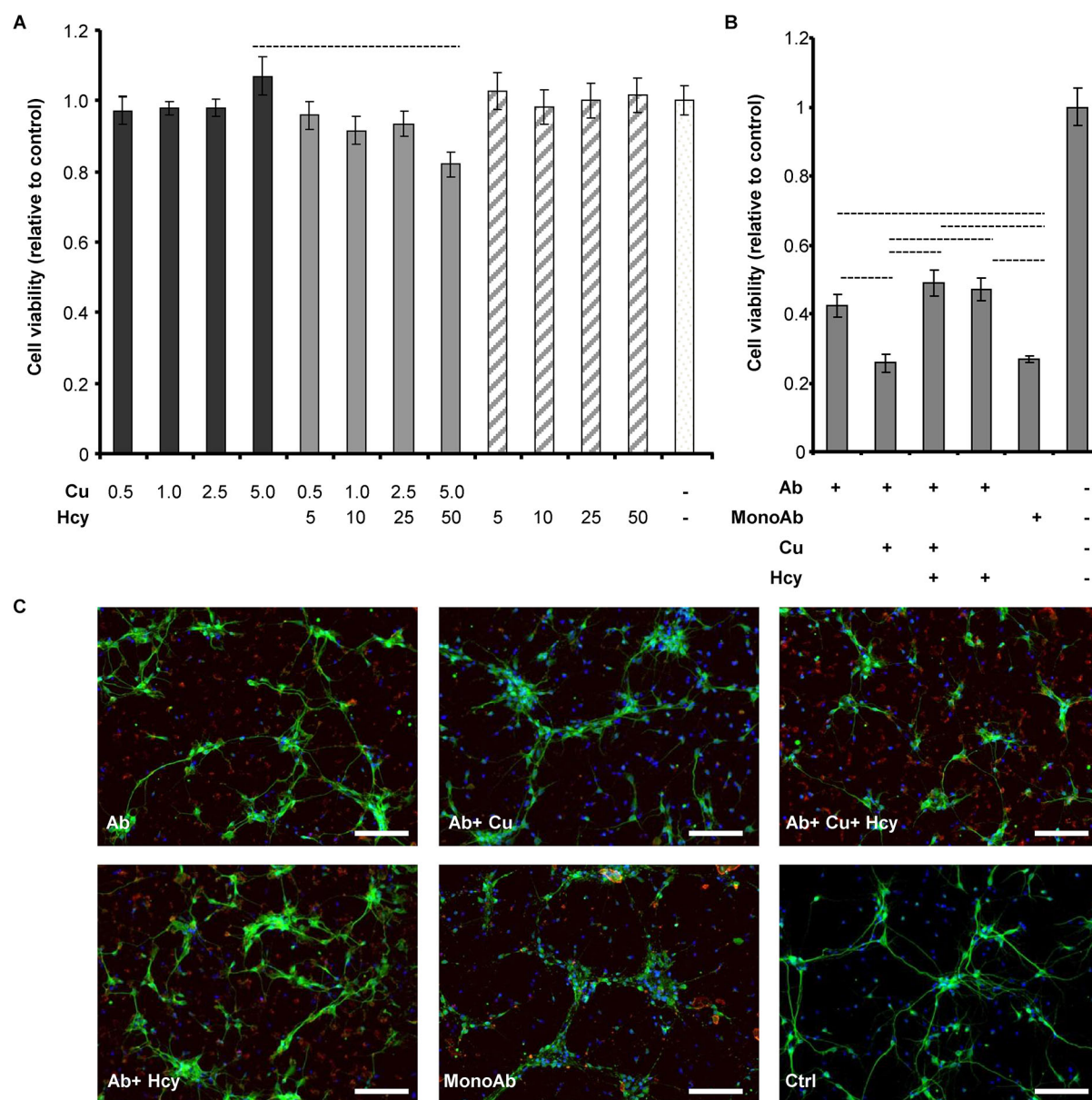


FIGURE 3 | Cell viability and morphology of rat primary cortical neurons after treatment with CuCl₂ and Hcy in the absence or presence of Aβ₄₂. Aggregations for viability and morphology studies were performed identically to previous aggregation assays, but lacking ThT due to its cytotoxicity. After 4 h agitation, samples were subsequently collected for the assessments. **(A)** Primary neurons were incubated for 72 h with increasing μM concentrations of CuCl₂ (dark gray), CuCl₂ + Hcy (light gray), or Hcy (stripes) without Aβ₄₂, to study their individual cytotoxicity. Control sample (aggregation assay reaction mixture) is visualized in the white column. **(B)** Effect of CuCl₂ and Hcy on

Aβ₄₂-induced toxicity. 5 μM Aβ₄₂; 5 μM Aβ₄₂ + 5 μM CuCl₂; 5 μM Aβ₄₂ + 5 μM CuCl₂ + 50 μM Hcy; 5 μM Aβ₄₂ + 50 μM Hcy were incubated on the cells for 72 h. As controls non-fibrillar 5 μM Aβ₄₂ (MonoAb) and aggregation assay reaction mixture without Aβ₄₂ were used. All values are relative to reaction mixture control sample ± S.D. **(C)** Immunofluorescent staining of primary cortical neurons after 24 h incubation. Antibody against neuronal marker, MAP2 (green), visualizes the changes of neuronal morphology; whereas anti-human APP (red) shows the Aβ aggregates and DAPI (blue) the cell nuclei. Concentrations were as indicated in **(B)**. Scale bar represents 100 μm.

Next we studied the toxicity of Aβ₄₂ fibrils formed in the presence of CuCl₂, Hcy or both (**Figure 3B**). Viability of primary neurons decreased to 40% after 72 h incubation with 5 μM Aβ₄₂ forming fibrils alone. Additional presence of 50 μM Hcy or 5 μM CuCl₂ plus 50 μM Hcy resulted in a slightly higher viability

of 50%. 5 μM Aβ₄₂ incubated in the presence of 5 μM CuCl₂ showed a significant increase in toxicity reducing cell viability to 25%. The same viability was observed after incubation with 5 μM monomeric Aβ. This shows that most likely CuCl₂ induces the formation of lower order oligomers or amorphous aggregates of

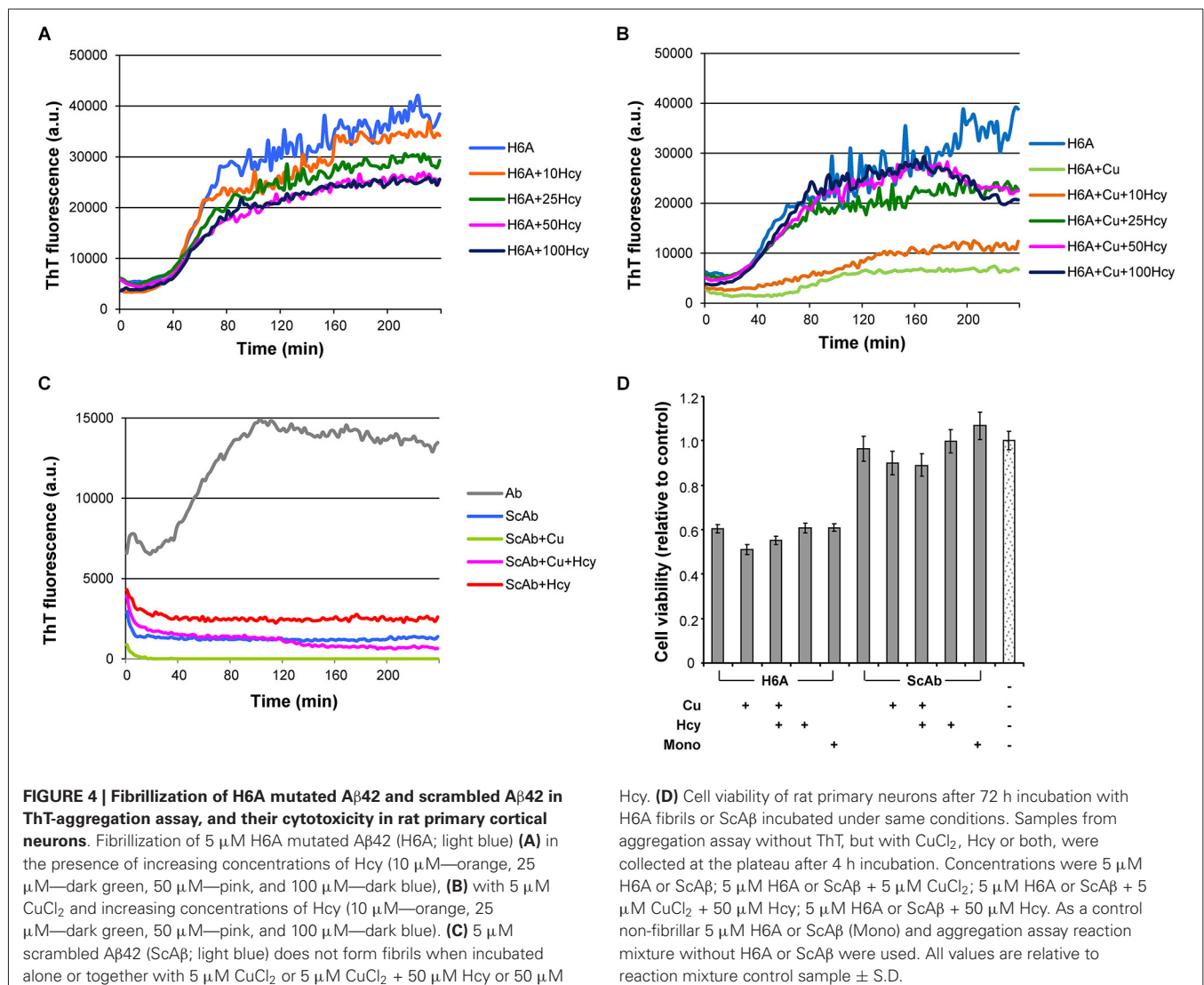
A β that have high cytotoxicity, whereas Hcy diminishes this effect and does not obviously contribute to cytotoxicity itself under the selected experimental conditions.

Observations from cytotoxicity studies were confirmed by morphological analysis of neurons treated with A β 42 aggregates collected after 4 h of aggregation without ThT (**Figure 3C**). Cells incubated with 5 μ M A β 42 plus 5 μ M CuCl₂ were shrunken and presented fewer neurites, resembling the morphology of cells treated with 5 μ M monomeric A β 42. In both, A β 42 plus CuCl₂ and monomeric A β 42 treated cells, also anti-APP staining patterns were similar visualizing fewer and smaller amyloid plaque-like structures. A β 42-stainings in other treatment conditions were similar to each other. Vehicle (without A β 42) serving as a negative control for anti-APP staining showed no such effects.

CuCl₂ HAS LIMITED EFFECTS ON THE AGGREGATION OF H6A MUTATED A β 42

Fibrillization of His6Ala-mutated A β 42 (H6A), which has low affinity to copper (Sacco et al., 2012), was studied to examine

the specificity of copper induced inhibition of A β 42 aggregation. Homocysteine only caused a minor concentration-dependent reduction in the maximum of ThT fluorescence in H6A fibrillization, which might have been due to unspecific variation in the ThT signal (**Figure 4A**). At Hcy concentrations higher than 50 μ M, aggregation curves remained unchanged. Addition of 5 μ M CuCl₂ to the aggregation reaction containing 5 μ M H6A inhibited its fibrillization leading to a longer lag phase, a decreased slope and plateau. Nonetheless, CuCl₂ was not able to completely prevent H6A fibrillization (**Figure 4B**). The effect of CuCl₂ on H6A was thus decreased compared to its effects on wild-type peptides. Increasing concentrations of Hcy (10–100 μ M) in the aggregation reaction of 5 μ M H6A plus 5 μ M CuCl₂ reversed the inhibitory effect of CuCl₂ on H6A fibrillization (**Figure 4B**). The concentration of Hcy needed to restore H6A fibril formation was smaller than in the case of wild-type A β 42. As H6A has a lower affinity to copper than A β 42, this underscores that there is a competition in binding of copper between A β 42 and Hcy as underlying mechanism of the interaction of Hcy and copper



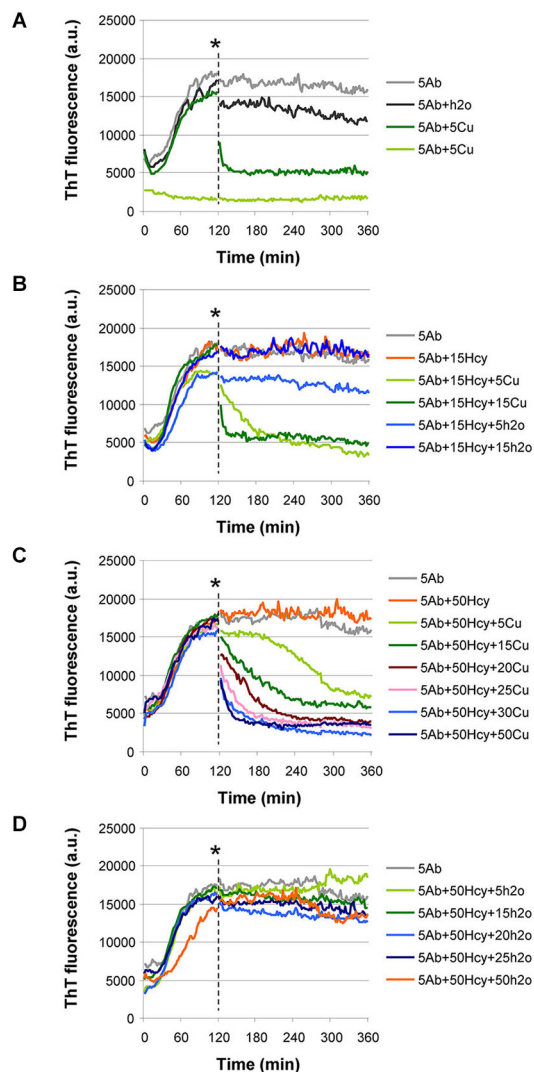


FIGURE 5 | A β 42 fibrils untangle by addition of CuCl₂ at timepoint 120 min in ThT aggregation assay. (A–C) CuCl₂ was added at the point indicated by an asterisk. **(A)** Addition of 5 μ M CuCl₂ caused a drastic reduction in ThT fluorescence when added to an aggregation reaction of 5 μ M A β 42 (dark green). Water, in equal volume, (H₂O, dark gray) did not induce reduction in ThT fluorescence. As controls, aggregation of 5 μ M A β 42 (light gray) and 5 μ M A β 42 + 5 μ M CuCl₂, added at the beginning of the reaction (light green), are shown. **(B)** When A β 42 fibrils are formed in the presence of 15 μ M Hcy (orange), their untangling after CuCl₂ addition depends on the molar ratio between CuCl₂ and Hcy (5 μ M CuCl₂—light green and 15 μ M CuCl₂—dark green). Water added in equal volumes to CuCl₂ did not change ThT fluorescence (light and dark blue). As a control aggregation of 5 μ M A β 42 (light gray) is shown. **(C)** After CuCl₂ addition A β 42 fibrils that were previously formed in high Hcy concentration (5 μ M A β 42 + 50 μ M Hcy) untangled. Degree of untangling was dependent on the molar ratio of A β 42 to added CuCl₂ and on the molar ratio of Hcy to added CuCl₂ concentration (5 CuCl—light green, 15 μ M CuCl₂—dark green, 20 μ M CuCl₂—purple, 25 μ M CuCl₂—light pink, 30 μ M CuCl₂—light blue, and 50 μ M CuCl₂—dark blue). **(D)** Water addition at timepoint 120 min did not induce changes in the ThT fluorescence of the reactions of 5 μ M A β 42 + 50 μ M Hcy. Water was added in equal volumes as CuCl₂ in panel (C).

on A β fibrillization. The increase in ThT fluorescence is specific to the formation of cross-pleated β -sheets. Accordingly, ThT

fluorescence of incubation mixtures containing scrambled A β 42 (ScA β), a peptide derivative of A β 42 that does not form fibrils, did not differ after addition of either 5 μ M CuCl₂, or 50 μ M Hcy or 5 μ M CuCl₂ plus 50 μ M Hcy confirming that the above described results did not result from artefacts induced by Hcy or copper in the incubation mixtures (Figure 4C).

TOXICITY OF A β 42, H6A AND ScA β TO PRIMARY CORTICAL NEURONS

In primary neuron cultures, fibrils of 5 μ M H6A, formed either in the absence or presence of 5 μ M CuCl₂, 50 μ M Hcy, or a combination of both, each reduced cell viability to approximately 60% after 72 h incubation (Figure 4D). Viability of neurons treated with 5 μ M ScA β , incubated in the absence or presence of 5 μ M CuCl₂, 50 μ M Hcy or both, remained at approximately 90%. The overall toxicity of the H6A was significantly higher than the one of ScA β , but lower than of A β 42.

ADDITION OF CuCl₂ TO ALREADY AGGREGATED SAMPLES UNTANGLED A β 42 FIBRILS

To study whether copper supplementation could be used to revert aggregation, we performed a simple aggregation assay in which CuCl₂ was added to an already fibrillized sample of either A β 42 alone or of A β 42 incubated in the presence of 15 μ M or 50 μ M Hcy. We selected these Hcy concentrations as they define the lower range of Hcy plasma concentrations in mild and intermediate hyperhomocysteinemia, respectively (Stanger et al., 2009).

The addition of 5 μ M CuCl₂ to a sample of 5 μ M A β 42 at time point 120 min drastically reduced ThT fluorescence, indicating untangling of A β 42 fibrils (Figure 5A) similar to the preventive effect of copper on fibrillization (Figure 2C). Only a minor reduction in ThT fluorescence was observed after addition of an equal volume of water (vehicle in which copper had been dissolved) to already fibrillized 5 μ M A β 42 at the same time point as negative control (Figures 5A,B). After addition of increasing CuCl₂ concentrations (5–15 μ M), A β 42 fibrils that had formed in the presence of 15 μ M or 50 μ M Hcy also untangled (Figures 5B,C). The decrease in ThT fluorescence depended on the ratio between copper and Hcy (Figures 5B,C). ThT fluorescence remained unchanged after vehicle additions (Figure 5D).

DISCUSSION

Hyperhomocysteinemia is a risk factor for AD, in which A β fibrillization plays an important role. Our study suggests that copper is a link between Hcy and A β . First, via a ThT assay and TEM, we confirmed that *in vitro* CuCl₂ prevents and reverts A β fibril formation (House et al., 2004; Bolognin et al., 2011; Chen et al., 2011; Figures 1A–C, 5A–C). Addition of CuCl₂ results in decreased A β fibril length and complexity i.e., lack of higher order aggregates (Figure 2C). The underlying mechanism is most likely connected to the ability of CuCl₂ to prevent the formation of A β 42 beta-sheets *in vitro* (Yoshiike et al., 2001; House et al., 2004), but due to the nature of ThT assay we cannot completely rule out the possibility of the formation of oligomers or amorphous aggregates. However, Hcy did not affect the fibrillar structure of A β as seen in ThT assay and in TEM images (Figures 1B, 2B).

Our ThT aggregation assay results of CuCl₂ and A β are in-line with other published results with similar experimental setup. It has been presented that in experimental conditions where fibril formation is fast, metal ions lower concentration of free peptide and thus inhibit fibrillization (Töugu et al., 2009). In conditions with slow fibril formation metal ions enhance fibril formation by metal-induced aggregates that can turn into fibrils (Sarell et al., 2010). Differences in experimental setup are also greatly influencing the outcome of fibrillization studies. This is summarized in a recent publication by Viles (2012) and shows multiple differences associated with changes in stoichiometry, used peptide preparation, concentration and study technique.

Cytotoxicity experiments performed in the absence of A β 42 showed that CuCl₂ or Hcy alone had no effect on cell viability (Figure 3A). In accordance to previous studies, cytotoxicity was elevated when cells were co-incubated with CuCl₂ and Hcy, confirming the toxicity of Hcy-copper-complexes (Figure 3A; White et al., 2001). In accordance with our ThT assay data and TEM images, toxicity of mature A β 42 fibrils to primary neurons was high, and A β fibrils formed in the presence of Hcy and CuCl₂ showed similar toxicity (Figures 1C, 2A–C, 3B,C). A β 42 co-incubated in 1:1 molar ratio with CuCl₂ showed the same level of toxicity as monomeric A β 42 underlining that CuCl₂ effectively inhibited the formation of bigger, more mature fibrils during ThT aggregation assays (Figures 2C, 3B; House et al., 2004; Chen et al., 2011). It has been reported that the fibrillar status of A β 42 affects its cytotoxicity in cultured neurons: small soluble A β 42 dimers and oligomers cause higher toxicity than bigger fibrillar forms (Klyubin et al., 2005; Lesné et al., 2006; Agnati et al., 2007; Ferreira et al., 2007; Haass and Selkoe, 2007; Ono et al., 2009).

As a certain time-span is required for A β preparations in contact with primary neurons to exert the cytotoxic properties, we cannot completely rule out that during the 72 h incubation a modification of the A β species can occur. However, as a result of our A β ThT aggregation assays we observed that the addition of CuCl₂ almost completely prevented the formation of higher order aggregates (sensitive to ThT fluorescence). As CuCl₂ itself did not have a measurable influence on cell viability, we infer that the observed cytotoxicity is due to A β monomers and potentially aggregates of lower molecular weight, such as A β di- and oligomers, A β -derived diffusible ligands or protofibrils (all probably being ThT fluorescence negative) that may have formed during the incubation period.

Copper has four coordinating ligands in human A β : 3N and 1O, which involve His6, His13, His14 and possibly Tyr10, carboxylate group of Asp1, the amide of Ala2, and the N-terminal amine. To date, no consensus of exact coordinating ligands exists. When single histidines were mutated to alanine, Hong et al. (2010) observed that His6 has three times higher copper binding constant than His13 or His14. His6 also requires less conformational changes upon copper binding, making it more entropy-favored. His6 was concluded to be ubiquitously involved in copper binding, accounting for 50% of the A β bound Cu(II). In our experiments, Hcy had only minor effect on the fibril formation of H6A mutant of A β 42 with reduced affinity to copper (Figure 4A). Also the effect of CuCl₂ was weaker in H6A

fibrillization, and the concentration of Hcy needed to restore H6A fibril formation was reduced compared to wild type A β 42 (Figures 4B, 1C). This allows the speculation that the amount of mature fibrils i.e., higher order aggregates in this sample was higher compared to the wild type A β 42. In accordance to this, H6A fibrils also showed less pronounced cytotoxicity in the presence of CuCl₂ (Figure 4D). The control, ScA β , expectedly showed no fibrils in ThT-assay (Figure 4C). Accordingly, ScA β was not cytotoxic and this was not influenced by incubation with CuCl₂ or CuCl₂ plus Hcy (Figure 4D). In ScA β the copper binding sites are lost, confirming that binding of copper to A β was the decisive mechanism for the observed differences in the ThT assay and the cytotoxicity experiments of the different combinations.

The addition of CuCl₂ to already fibrillized A β led to untangling of fibrils (Figures 5A–C). This does not necessarily mean that copper is relevant for A β fibrillization *in vivo*, however, mice with defective copper transport have decreased brain copper levels together with increased amounts of amyloid plaques. When these mice are crossed with Wilson's disease mouse model, the offspring have increased brain copper levels, less amyloid plaques and a longer life span (Phinney et al., 2003). When copper sulphate was added to the drinking water of mice susceptible to amyloid accumulation, less accumulation was observed (Bayer et al., 2003). In patients with mild to moderate AD, plasma copper negatively correlates with cognitive abilities (Pajonk et al., 2005; Kessler et al., 2006). In addition, AD patients show elevated serum levels of free copper (serum copper not bound to ceruloplasmin) (Squitti et al., 2004, 2005, 2006, 2009, 2011; Capo et al., 2008) while autopsy samples of hippocampus and amygdala from AD patients showed generally reduced copper contents (Deibel et al., 1996; Klevay, 2008).

In contrast, in the amyloid plaques, copper concentration can be as high as 400 μ M, although normal brain extracellular concentration is 0.2–1.7 μ M (Gutteridge, 1984; Kardos et al., 1989; Linder and Hagezh-Azam, 1996; Lovell et al., 1998; Schumann et al., 2002; White et al., 2004; Squitti et al., 2006). This data seems to be in contrast to our observation that CuCl₂ prevents amyloid fibril formation. However, in our experiments, CuCl₂ alone prevented and reverted aggregation, whereas Hcy plus CuCl₂ did not. Thus, it would be interesting to analyze whether copper in amyloid plaques of AD patients is bound to Hcy or other molecules. Moreover, the inhibitory effect of CuCl₂ on fibrillization was concentration-dependent (Figures 5B,C). Thus, one may speculate that in the copper-rich plaques of AD patients, copper levels may not have reached the necessary concentration.

Serum Hcy concentrations over 14 μ M are an independent risk factor for the development of AD (Seshadri et al., 2002). In our study, the addition of Hcy alone did not change A β 42 fibril formation (Figure 1B), although the addition of Hcy slightly reduced A β 42 toxicity *in vitro* (Figure 3B). In co-incubation experiments, Hcy concentration-dependently reduced the inhibitory effects of CuCl₂ on A β 42 fibrillization suggesting that Hcy and A β compete for copper binding, i.e., homocysteine-bound copper has reduced or no effects on A β 42 fibril formation (Figure 1C). Two different complexes are possible between Hcy and copper, showing molar ratios of 1:1 or \leq 1:3

(Apostolova et al., 2003). Similarly, the majority of the copper-A β complexes form with a 1:1 stoichiometry at physiological pH (Karr et al., 2005; Syme and Viles, 2006; Töugu et al., 2008; Faller and Hureau, 2009). For H6A, Hcy was more effective in neutralizing the effect of CuCl₂ on fibril formation confirming that CuCl₂ is less effective in preventing fibrillization of this mutated peptide due to its reduced affinity to copper (**Figure 4B**).

In summary, this study shows that both Hcy and A β 42 bind and compete for copper. Copper prevents and reverts fibril formation by binding to A β 42 and thereby increases A β toxicity. Homocysteine builds toxic complexes with copper and concentration-dependently prevents the effects of copper on A β 42 fibrillization. In the presence of A β 42, neurotoxicity of copper is reduced giving rise to the speculation that one physiological A β function might be the prevention of copper neurotoxicity. Due to complex building, hyperhomocysteinemia reduces the availability of free copper, which in the light of our results, likely increases amyloid plaque formation. Acute presence of high copper concentrations untangle aggregates leading to high concentrations of mono- or oligomeric A β 42-copper complexes causing marked neurotoxicity. The analysis of the interaction between A β 42, copper and Hcy in patients may lead to novel therapeutic strategies in the prevention and treatment of AD.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 June 2014; accepted: 20 August 2014; published online: 08 September 2014.

Citation: Keskitalo S, Farkas M, Hanenberg M, Szodorai A, Kulic L, Semmler A, Weller M, Nitsch RM and Linnebank M (2014) Reciprocal modulation of A β 42 aggregation by copper and homocysteine. *Front. Aging Neurosci.* 6:237. doi: 10.3389/fnagi.2014.00237

This article was submitted to the journal *Frontiers in Aging Neuroscience*.

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